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(54) **Sugar Coated Reagents for Solid Phase Immunoassay**

(57) An immunoadsorbent coated onto a solid support can be stabilized by employing a sugar coating. Stabilization according to the disclosure will permit dry handling and storage of the coated support. Reagents for use in immunoassay comprise sugar-coated hepatitis antigen or antibody fixed directly or indirectly to for example polystyrene beads.

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SPECIFICATION

Sugar Coated Reagents for Solid Phase Immunoassay

This invention discloses an improvement in solid phase immunoassay methods for the detection and determination of antigens and antibodies, especially those relating to hepatitis.

Hepatitis A is usually characterized by a short incubation period of two to six weeks, mild prodromata and a relatively mild clinical illness. The disease is generally transmitted by contaminated food or liquids, but has also been shown to be transmitted by systemic inoculation. Hepatitis A was previously called "infectious hepatitis". In the United States the number of reported cases of non-B viral hepatitis, generally considered to be mainly hepatitis A, is over 50,000 annually and estimates of actual U.S. incidences are as high as 1.2 million.

Because there has not been a convenient, specific assay for hepatitis A antigen (HAVAg) or its antibody (anti-HAV), diagnosis has had to relay on clinical symptoms, association with a point or source of outbreak, patient history, tests for liver function and the absence of markers for hepatitis B infection.

Two recent discoveries have encouraged the development of specific tests to indicate contraction or exposure to the disease: Major efforts at identifying animal models for studying hepatitis A culminated by finding, in 1973, that marmosets, and later chimps, were susceptible to hepatitis A virus infection (HAV); and the discovery of a virus-like particle 27 nm in diameter associated with hepatitis A by Feinstone *et al.*, in 1973. Using the technique of immune electron microscopy (IEM), Feinstone *et al.*, observed these particles in stool extracts of patients with acute phase hepatitis A and showed that they were aggregated by convalescent sera but not by pre-infection sera from the same patients. Identical HAV particles were subsequently identified in serum and liver of infected primates.

These first elaborate biological and IEM techniques were soon followed by more practical immunologic methods for detection of HAAg and anti-HAV. Provost *et al.* described a complement fixation test and demonstrated its efficacy for anti-HAV detection; Miller *et al* and Moritzugu described sensitive immune adherence (IAHA) tests, also applicable mainly to anti-HAV detection. Hollinger *et al* and Purcell *et al* have described radioimmunoassay (RIA) procedures with high sensitivity for the detection of both HAVAg and anti-HAV in biological specimens. IAHA and RIA tests for anti-HAV appear to be the two most useful procedures for laboratory applications. Dienstag *et al* have made comparative studies of IAHA and RIA and have found that the two tests compare well in terms of sensitivity and specificity. Another study further substantiated this finding but is was noted that RIA detects other earlier antibodies specific for anti-HAV in addition to those detected by IAHA. (Bradley *et al.*, J. Clin. Microbiol. 5:521—530, 1977).

Heptatis B infection is generally transmitted by blood products or contaminated instruments such as needles, but it may also be transmitted by a fecal-oral connection. Previously a hepatitis B infection was associated with an incubation period ranging from six weeks to six months. Recently, however, incubation periods as short as two weeks have been documented. The illness may be mild or asymptomatic, but if symptomatic, manifestations may be especially severe. Prodromata may include arthralgias, arthritis, rash, fever, anorexia, fatigue and pruritis with or without jaundice.

At least two distinct antigen-antibody systems can be associated with hepatitis B: the surface (HB_sAg:anti-HB_s) and the "core" (HB_cAg:anti-HB_c). The hepatitis B surface antigen (HB_sAg) found in the blood as 22 nm spheres and as elongated tubules which are 22 nm in diameter and variable in length are believed to represent the coat protein of the hepatitis B virus. A 42 nm particle containing DNA and a DNA polymerase is considered to represent the infectious virus (Dane particle). In detergents, the Dane particle is degraded to a 26 nm electron dense core, HB_cAg. The latter is seen in nuclei of hepatocytes of patients with serum hepatitis during the acute infection stage. Thus, patients with viral hepatitis type B might be expected to produce antibodies to the protein coat surface antigen (anti-HB_s), and also to the protein core (anti-HB_c).

Anti-HB_c appears 12—20 weeks after exposure, often accompanying antigenemia (HB_sAg), at the height of liver dysfunction and long before the appearance of anti-HB_s. Anti-HB_c is generally associated with prolonged circulation of HB_sAg suggesting that anti-HB_c is produced in response to the active replication of the virus.

HB_sAg, anti-HB_s, and anti-HB_c exist singly in serum or may coexist in combination in an individual specimen. All three are useful to gauge the course of hepatitis B virus infection.

Diagnosis of hepatitis B has also included various tests, such as immunodiffusion or agar-gel diffusion, counter-electrophoresis, complement fixation, hemmagglutination and radioimmunoassay.

Because of its simplicity and sensitivity, the diagnostic system of preference for detecting hepatitis A and B antigens and antibodies is solid phase radioimmunoassay (RIA). This procedure permits simple and rapid separation of the bound and unbound immunoreactants utilized in most immunoassays. A disadvantage to solid phase RIA for commercial hepatitis diagnosis, however, has been the relative instability of hepatitis immunoreactants when coated directly or indirectly to the solid phase material.

Garrison *et al* U.S. Patent No. 3,790,663 is directed to antibodies affixed to a solid support and useful in the detection of antigens. The disclosed invention is directed to an improved reagent

comprising a sugar coated immunoadsorbent affixed to a solid support which is useful for the detection of antigens and antibodies, primarily those related to hepatitis A and B. The teaching of Garrison *et al* does not encompass the claimed reagents and, of course, it does not suggest the reagent stability demonstrated by the sugar coated reagents as demonstrated in assays set forth below.

It is an object of the present invention to provide a reagent for the detection and determination of immunoreactants such as antibodies and antigens in immunoassays. It is an additional object of this invention to provide an improved reagent useful in an immunoassay for the diagnosis of hepatitis by disclosing the preparation and use of a solid phase reagent that is stable over a period of months rather than hours. The storage stability of the disclosed reagents will obviate the need to use a freshly prepared solid phase coated with an immunoreactant and thereby make the use of a solid phase immunoassay practical in routine clinical determinations.

This invention relates to a storage stable reagent useful in immunoassays for the detection and determination of antigens and antibodies. These reagents will ideally consist of a solid support that has been coated either directly or indirectly with an antigen or antibody and stabilized with a sugar coating to impart a storage capability.

An indirect application of an antibody or antigen to a solid support generally involves the procedure in which the solid support is precoated with antigen or antibody to potentiate the adherence of the corresponding antibody or antigen. For example, if the solid support is to be ultimately coated with hepatitis A antigen, the support might be precoated with hepatitis A antibody.

In either the direct or indirect attachment of the antigen or antibody to the solid support, it is desirable to preserve either the avidity of the antibody or the antigenicity of the antigen. The disclosed method provides for a method of maintaining avidity and antigenicity of the exposed immunoadsorbent by applying a sugar coat.

This invention has solved the stability problem encountered when antigen and antibodies are coated directly or indirectly to solid phase material. It has been observed that the exposed antibody or antigen will lose its avidity or antigenicity in a matter of hours and be essentially useless in an assay for the detection of the corresponding antibodies or antigens. One solution to this problem has been to maintain the coated solid support in a wet or moist condition by storing in a buffered saline solution. This does an adequate job of maintaining avidity and antigenicity, but the inconvenience and expense of sustaining this environment is readily apparent.

In an immunoassay for the detection of antibodies, an antigen having an affinity for the antibodies is affixed to an easily manipulated solid support. If they can be obtained in a reasonably pure condition to assure adequate concentration, antigens may be affixed directly to the surface of the solid support. Also, some antigens will adhere to the surface of the solid support more readily than others. Those demonstrating less affinity for the surface of the support can be affixed by employing an antibody precoat. In that instance, the solid support is coated with antibody in a conventional manner and then exposed to a source of antigens.

Generally, antigens adhere more readily to the affixed antibody and the antigen need not be purified before exposure to the antibody coat. The affinity between the antibody and antigen assures selective retention of antigenic material, and debris accompanying the antigen may be washed away.

The solid phase support material contemplated by this disclosure may include beads, tubes, wells and rods which may be fabricated from a variety of materials including glass, metal or plastic. The preferred embodiment of this invention employs a polystyrene bead. This material is readily available, easy to coat with immunoadsorbents and easy to manipulate. It is essential to remember that the invention is manifested in the stability of the immunoadsorbent affixed to the solid support, not with the solid support alone.

The immunoadsorbents contemplated as within the scope of this invention include all antibodies and antigens which exhibit immunoreactive properties. The most preferred embodiments of the present invention feature antigens or antibodies having an affinity for the antibodies and antigens of either hepatities A or B employed as immunoadsorbents and affixed to a polystyrene bead either directly or indirectly according to the method set forth below.

The sugar coating contemplated to be within the scope of this invention includes mono-, di- and polysaccharides. Although the examples set forth below will demonstrate the particular effectiveness of sucrose, other sugars have been formulated and applied to antigen coated supports and also have been found to be effective at preserving avidity and antigenicity. For example, the following sugar solutions were prepared:

Table I

xylitol	10% in PBS (Phosphate Buffered Saline)
lactose	10% in PBS
glucose	10% in PBS
mannitol	10% in PBS
PBS	
sorbitol	10% in PBS
dextran	10% in PBS

Polystyrene beads pre-coated with hepatitis A antibody and subsequently exposed to a source of A antigen were washed three times in PBS. They were then soaked in the formulations of Table I for 30 minutes at room temperature. Except for a few of the beads soaking in the PBS only, all beads were removed and allowed to dry at room temperature overnight. In the morning, they were placed in an incubator at 37°C for 2 hours. The assay for antigenicity was conducted using radiolabeled antibody on 3 negative controls and 3 positive controls for each set.

The ratio of the average counts per minute for the negative to the positive controls gives an indication of antigen activity remaining on the bead. The following table lists these ratios indicating the relative effectiveness of the sugar coatings employed.

Table II

1.	PBS wet	36.9
2.	glucose	32.7
3.	lactose	29.8
4.	sucrose	28.5
5.	xylitol	26.8
6.	dextran	26.8
7.	sorbitol	25.9
8.	mannitol	22.4
9.	PBS dry	7.9

The foregoing indicates that a variety of sugars will serve to coat, protect and preserve the activity of an antigen coated solid support.

The following examples will demonstrate further the utility of the disclosed invention:

Example I.

Antiserum containing anti-HAV was diluted 1:500 to 1:6000 with 0.01 M Tris buffer at pH 9.5. To this diluent was added a polystyrene bead of approximately 0.7 cm in diameter. The coating process was allowed to take place at room temperature for approximately two hours. The beads were then washed in the Tris buffer and submitted to either liver or fecal extracts which were positive for HAV-Ag and which had been diluted 1:5 to 1:100 with 0.01 M phosphate with 0.3 M saline to give a buffer (PBS) at pH 7.5. The HAV-Ag may be inactivated prior to use by formalin and heat treatment by conventional methods. No purification of the HAV-Ag containing extract is necessary beyond centrifugal clarification. The beads were allowed to become coated with HAV-Ag which binds the beads by way of the anti-HAV precoat. This HAV-Ag coating process was allowed to proceed at room temperature for 24 hours. The beads were then washed in PBS in which the beads are stable and stored until further use. To obtain stable dry beads, the PBS washed beads were coated with 5% sucrose solution at room temperature for approximately 30 minutes and then air dried.

Preparation of ^{125}I-labeled Antibody Reagent

^{125}I-labeled antibody (anti-HAV) was prepared by a conventional method and diluted into 50% fetal calf serum containing PBS, 2% normal human serum and 0.2% Tween —20 and 0.005 M, 45°C for 24—48 hours. If a higher temperature were used such as 56°C, the incubation could be cut to 0.5—3 hours.

Assay Procedure

Serum or recalcified plasma was preferred as the sample to be tested for hepatitis A antibody (anti-HAV).

The assay for the detection of antibody to hepatitis A antigen is based on the principle of competitive binding of serum anti-HAV with radioactive labeled anti-HAV to hepatitis A antigen (HAV-Ag). In a test tray, anti-HAV ^{125}I was mixed with the patient's serum and a stable solid phase reagent on which the HAV-Ag has been bound, was then added. After incubation overnight at room temperature or for 4 hours at 45°C and a subsequent washing, the count rate of the bead was determined in a gamma radiation counter and recorded.

A count rate higher than the established cut-off value was negative for antibody while a low count would indicate competitive binding between added radiolabeled antibody and indogenous antibody in the serum. The presence of anti-HAV can also be determined by calculating the percent neutralization of the patient specimen compared to the test controls. Percent neutralizations greater than the 50% cut-off value indicate the presence of antibody by the same logic as outlined above.

Example II

Preparation of Stable Solid Phase Reagent

Dane particle preparations of various stages of purity were treated with 2-mercaptoethanol (0.30—0.75%) and a non-ionic detergent, such as Triton X-100 or Nonidet P-40, in a concentration of about 1.0 to 2.5% at 37°C for one hour. The purpose of this treatment was to remove the lipoprotein

coat and to release the core antigen of the Dane particles. The above conditions for the treatment are preferred, but they may be varied without adverse effect. After the treatment, the mixture was appropriately diluted with a buffer solution; for example, 0.01 M Tris-HCl, pH 7.1, in physiological saline solution containing 0.001 M EDTA. The solution was used immediately to coat the solid surface of objects such as beads, tubes or wells made of plastic or glass. Dane cores (HB_c Wag) prepared in this manner are very "sticky", and easily attach to solid surfaces by adsorption. When preparations of Dane particles are grossly impure and contain high levels of extraneous proteins, it is necessary to precoat the solid objects with anti-HB_c before reacting with Dane cores, as above. With my preparations, when polystyrene beads were incubated with the Dane core solution for 24 to 72 hours, there was more Dane core on plain beads than on anti-HB_c precoated beads, especially when very low concentrations of Dane core were used. It has been discovered that in the coating solution, the detergent concentration should be very low (preferably lower than 0.005%) if Dane cores are coated directly on solid surfaces.

To stabilize the resulting HB_cAg coated bead, a 5—10% sucrose solution was employed. The HB_cAg bead was incubated in the said sucrose solution for about 20 minutes at room temperature and then air dried.

Preparation of ^{125}I-labeled Antibody Reagent Example

^{125}I-labeled Dane core antibody (anti-HB_c) to HB_cAg was prepared by conventional methods and diluted into 0.005 M Tris with 0.04 M EDTA buffer pH 7.3, containing 50% fetal calf serum and 2% recalcified normal human plasma, and 0.4% Tween —20.

Assay Procedure

Serum or recalcified plasma was preferred as the sample to be tested for hepatitis B antibody (anti-HB_c). The test for the detection of anti-HB_c is based on the principle of competitive binding to hepatitis B core antigen (HB_cAg) coated beads between ^{125}I-radiolabeled anti-HB_c and anti-HB_c present in the patient's specimen. In a test tray, the patient specimen is incubated together with ^{125}I-radiolabeled anti-HB_c and an HB_cAg coated bead for approximately 20 hours at room temperature. After incubation and washing, the count rate of the bead is determined in a suitable gamma radiation detector and recorded. A count rate higher than the established cut-off value is indicative of the absence of anti-HB_c or presence of undetectable levels of anti-HB_c in the specimen while a count rate lower than the established cut-off value is indicative of the presence of anti-HB_c in the serum.

The same procedure for stabilization of solid phase outlined above may be followed where hepatitis B surface antigen is coated directly or indirectly to the solid phase material and the RIA is for antibody to hepatitis B surface antigen. The two procedures in the examples depend primarily on the purity of the antigen to be coated on the solid phase. Highly pure antigen may be coated directly on the bead without the necessity of pre-coating with antibody to that antigen.

Example III

Guinea pig antiserum containing anti-HB_s was diluted 1:1750 in phosphate buffered saline (PBS, 0.01 M sodium phosphate, 0.15 M sodium chloride, pH 7.2). This solution was added to a flask containing polystyrene beads of diameter 0.64 cm. This coating solution, containing the beads, was warmed to 45°C by immersion into a 45°C water bath for 2 hours. The beads were then washed twice with PBS (which was at room temperature). The beads were then coated with a solution of PBS containing 2% sucrose at room temperature for approximately 15 minutes. The beads were then air dried.

Using this procedure, additional beads were similarly coated with 2% lactose, 2% glucose and PBS alone by soaking for approximately 15 minutes. All beads were then air dried.

Example IV

A radioimmunoassay for the detection of hepatitis B surface antigen was conducted using radiolabeled antibody on negative controls, samples containing HB_sAg/ad antigen and samples containing HB_sAg/ay antigen. The ratio of counts in the antigen-containing samples to those in the negative control samples is listed in the following table for polystryene beads coated with the various sugars:

Table III

Sugar Used in Bead Coating	*Ratio ad/Neg. Control Beads Stored at*		*Ratio ay/Neg. Control Beads Stored at*	
	4°C	*3d—45°C*	*4°C*	*3d—45°C*
Lactose	36.3	36.1	30.4	30.1
Sucrose	33.6	38.3	31.7	31.9
Glucose	37.2	37.9	31.3	31.0
(PBS only)				
No Sugar	24.5	21.0	15.1	9.6
Sucrose (in water)	36.1	31.0	28.1	28.5

The data in Table III indicates that a variety of sugars will serve to coat, protect, and preserve the activity of an antibody-coated solid support. The data from Table III also shows that antibody-coated beads lacking the protective sugar coating have reduced activity against the HB_sAg/ad and HB_sAg/ay antigens after heat stress, and activity against the HB_sAg/ay antigen is reduced even before heat stress.

Example V

An enzyme immunoassay for the detection of hepatitis B surface antigen was conducted using anti-hepatitis B surface antigen-peroxidase conjugate. Samples consisted of negative controls, samples containing HB_sAg/ad antigen and samples containing HB_sAg/ay antigen. The difference of the optical density at 492 nm in the antigen-containing samples minus that in the negative control samples is listed in the following table for polystyrene beads coated with the various sugars:

Table IV

Sugar Used in Bead Coating	*Ad minus Neg. Control Beads Stored at* 4°C	*Ad minus Neg. Control Beads Stored at* 3d—45°C	*Ay minus Neg. Control Beads Stored at* 4°C	*Ay minus Neg. Control Beads Stored at* 3d—45°C
Lactose	0.520	0.488	0.426	0.390
Sucrose	0.486	0.527	0.401	0.396
Glucose	0.479	0.503	0.413	0.365
(PBS only) No Sugar	0.339	0.257	0.217	0.121
Sucrose (in water)	0.513	0.420	0.496	0.374

The data in Table IV shows that a variety of sugars will serve to coat, protect, and preserve the activity of an antibody-coated solid support. The data from Table IV also shows that antibody-coated beads, lacking the protective sugar coating have reduced activity against the HB_sAg/ay antigen, before and after heat stress of the beads.

Claims

1. A stable reagent useful in the performance of immunoassays which comprises a sugar coated immunoadsorbent affixed to a solid support.
2. A stable reagent according to Claim 1 wherein the immunoadsorbent is an antigen.
3. A stable reagent according to Claim 2 wherein the antigen is selected from the group consisting of hepatitis A antigen, hepatitis B surface antigen, hepatitis B core antigen and hepatitise antigen.
4. A stable reagent according to Claim 1 wherein the immunoadsorbent is an antibody.
5. A stable reagent according to Claim 4 wherein the antibody is selected from the group consisting of hepatitis A antibody, hepatitis B surface antibody, hepatitis B core antibody and hepatitis e antibody.
6. A stable reagent useful in the detection and determination of antibodies in an unknown sample which comprises a sugar coated antigen affixed to a solid support.
7. A stable reagent useful in the detection and determination of antibodies in an unknown sample which comprises a sugar coated antigen affixed to an antibody coated solid support.
8. A stable reagent useful in the detection and determination of antigens in an unknown sample which comprises a sugar coated antibody affixed to a solid support.
9. A stable reagent useful in the detection and determination of antigens in an unknown sample which comprises a sugar coated antibody affixed to an antigen coated solid support.
10. A stable reagent useful in an immunoassay for the detection and determination of hepatitis antibody which comprises a sugar coated hepatitis antigen affixed to a plastic solid support.
11. A stable reagent useful in the detection and determination of hepatitis A antibody in an unknown sample which comprises a sugar coated hepatitis A antigen affixed to a hepatitis A antibody coated solid support.
12. The reagent according to Claim 11 wherein the solid support is a polystyrene bead.
13. A stable reagent useful in the detection and determination of hepatitis B core antigen in an unknown sample which comprises a sugar coated hepatitis B core antibody affixed to a solid support.
14. A stable reagent useful in the detection and determination of hepatitis B surface antigen in an unknown sample which comprises a sugar coated hepatitis B surface antibody affixed to a solid support.

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